Mutations of Ser-23 of the α1 subunit of the rat Na^+/K^+-ATPase to negatively charged amino acid residues mimic the functional effect of PKC-mediated phosphorylation

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Abstract **The Na^+/K^+-ATPase is a target protein for protein kinase C (PKC). The PKC-mediated phosphorylation of the rat α1 subunit at Ser-23 results in the inhibition of its transport function. To understand the molecular basis of the inhibition by PKC, the Ser-23 in the rat α1 subunit has been replaced by negatively (Asp, Glu) or positively (Lys) charged, or uncharged (Gln, Ala) residues, and the mutants were expressed in *Xenopus* oocytes. Ouabain-specific ^{86}Rb uptake and pump-generated current as well as sensitivity to ouabain and to external K^+ have been investigated. When Ser-23 was replaced by the negatively charged residues, transport function was inhibited, and simultaneously synthesis of the α subunits was enhanced. In addition, if Ser-23 was substituted by Glu, the K_I value for inhibition of transport by ouabain was drastically increased from 46.5 μM to 1.05 mM. The data suggest that insertion of a negative charge within the N-terminus of α subunit of the Na^+/K^+-ATPase due to phosphorylation of Ser-23 plays an important role in the PKC-mediated inhibition of transport function.**

Key words: Na^+/K^+-ATPase; PKC; Posttranslational modification; Mimicry; Phosphorylation; Regulation

1. Introduction

The Na^+/K^+-ATPase (E.C. 3.6.1.37) belongs to the P-type ATPases, which are catalytically phosphorylated at an aspartic acid residue, and maintains electrochemical gradients of Na^+ and K^+ across the plasma membrane of animal cells. Recently, it has been demonstrated that the Na^+/K^+-ATPase is a target protein for PKC [1–4] and Ser-23 in the rat α1 subunit has been shown to be a phosphorylation site [5–8]. PKC-mediated phosphorylation of Ser-23 results in inhibition of the transport function of the Na^+/K^+-ATPase [7–9]. Covalent modification of Ser and Thr residues by phosphoryl groups is a universal mechanism, which leads to dramatic changes in functional characteristics of phosphorylated proteins. However, little is known why the formation of phosphorylated residues has such drastic consequences. Since the binding of PO_4^{3-} brings net negative charges to the polypeptide side chain, electrostatic interactions may be considered as a possible microscopic mechanism. The PKC phosphorylation site of α1 subunit of the Na^+/K^+-ATPase is located within the positively charged lysine cluster in the N-terminus, which has been shown to play an important role in regulation of cation translocation [10–15]. This raises the question whether PKC-mediated inhibition of transport is due to the insertion of the negatively charged phosphoryl group within the positively charged K-cluster.

The objective of our work was to investigate whether substitutions of Ser-23 of the rat α1 subunit by negatively charged amino acids may reproduce the changes resulting from the covalent modification of the protein at this position by phosphorylation. Furthermore, the influence of uncharged and positively charged amino acid residues at position 23 was analysed.

Our results show that replacement of Ser-23 by the acidic residues Asp and Glu mimics PKC-mediated inhibition of the transport function of the Na^+/K^+-ATPase and leads to an enhanced synthesis of the α subunit protein.

2. Materials and methods

2.1. Construction of mutants

Rat α1 subunits were modified by site-directed mutagenesis as described previously [8] using the Altered Sites in vitro mutagenesis kit (Promega). Mutations of Ser-23 to Glu, Gln, Lys, Ala and Asp were obtained by annealing the following phosphorylated antisense oligonucleotides to single stranded pALTER1-rα1: S23E: 5′-T CGC CTT CTT **TTC** CTT CTT GTC CCC ATG TTC TGA TA*C* ***TGC AG***C GGG CTC AT-3′; S23Q: 5′-TT CGC CTT CTT **TTG** CTT CTT GTC CCC ATG TTC TGA TA*C* ***TGC AG***C GGG CTC AT-3′; S23K: 5′-TT CGC CTT CTT **TTT** CTT CTT GTC CCC ATG TTC TGA TA*C* ***TGC AG***C GGG CTC AT-3′; S23A: 5′-TT C*GC CTT CTT* ***GGC*** CTT CTT GTC C-3′; S23D: 5′-TT CGC CTT CTT **GTC** CTT CTT GTC C-3′.

Each substituted coding tripple is underlined, bold letters indicate changes of nucleic acid bases. The restriction sites introduced simultaneously are marked by italics, they do not alter the coded protein furthermore. Synthesis of mutated plasmid strands was performed with T4-DNA-polymerase and T4-DNA-ligase. The heteroduplex DNA obtained was used to transform *E. coli BMH 71–18 mutS*, which is defective in mismatch repair. Plasmid DNA isolated from *E. coli BMH 71–18 mutS* was used to transform *E. coli DH5α*. Mutated plasmids isolated from *E. coli DH5α* clones were identified either by restriction analysis or by DNA sequencing. To verify the mutations, DNA from selected clones was sequenced by the dideoxy method and then was used for cRNA synthesis. Capped cRNA was synthesised with SP6-RNA-polymerase (Ambion, Austin, TX, USA) and chromatographically purified.

2.2. Oocytes

Prophase-arrested defolliculated oocytes (stage V–VI) obtained from *Xenopus laevis* were injected with 46 nl of cRNA (1 mg/ml) for the wild-type or mutated α subunits of rat α1 isoforms and rat β subunit (mol/mol ratio). Injected oocytes and non-injected controls

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of the same batch were incubated at 19°C in oocytes's Ringer solution (in mM): NaCl 90, KCl 1, $CaCl_2$ 1, $MgCl_2$ 1, and HEPES 10 (pH 7.4) supplemented with 50 mg/l gentamycin for 2–3 days to allow expression.

2.3. Measurements of $^{86}Rb^+$ uptake and pump-generated currents

Before the measurements cells were loaded with Na^+ by incubation in a K^+-free Na^+-loading solution containing (in mM): Na-citrate 2.5, NaCl 110, and MOPS 5 (pH 7.6) for 45–60 min, and then incubated for equilibration in a post-loading solution (in mM): NaCl 100, $CaCl_2$ 1, $BaCl_2$ 5, (TEA)Cl 20, and MOPS 5 (pH 7.6). To eliminate indirect voltage-dependent changes of the Na^+/K^+-ATPase [16] during measurements of $^{86}Rb^+$ uptake, cells were incubated for 12 min in Na^+-free test solution (in mM): TMA 90, (TEA)Cl 20, $BaCl_2$ 10, $NiCl_2$ 5, MOPS 5 (pH 7.4), RbCl 10 and $^{86}Rb^+$ (925 kBq/ml) with or without 10 mM ouabain and then washed with Rb^+-free test solution. Ouabain-sensitive $^{86}Rb^+$ uptake was determined as a difference between the uptakes in solutions without ouabain and with 10 mM ouabain. For estimation of the effects of PKC on transport activity, 0.5 ng of catalytic domain of PKC, PKM (Biomol, Hamburg, Germany) was injected into the Na^+-loaded oocytes 30 min before the measurements. Pump-mediated currents were determined under voltage clamp as the component of membrane current activated by 10 mM external K^+. To block K^+-channels, 5 mM $BaCl_2$ and 20 mM (TEA)Cl were added. Measurements were performed at a holding potential of −30 mV. The test solutions contained, instead of Rb^+ and $^{86}Rb^+$, different concentrations of K^+ as indicated in Fig. 3.

2.4. Triton X-100 extracts and Western blot

For preparation of Triton extracts, five oocytes were disrupted by passing them through Eppendorf pipette tips in homogenisation buffer (HBO, 10 μl/oocyte) containing (in mM): Tris-HCl 20 (pH 7.4), $MgCl_2$ 5, EDTA 1, NaCl 100, KCl 10, DTT 1, PMSF 1, Triton X-100 1%, and 5 μg/ml each of leupeptin, pepstatin and antipain. Triton extracts, SDS-PAGE and Western blots were obtained as described previously [8]. α subunits were immunostained with anti-α1 or anti-α2 antibodies (a generous gift of Dr. O. Hansen, Denmark [17]) and visualised with secondary alkaline phosphatase-conjugated antibody (Biozol, Eching, Germany). Quantification of the immunoblots was done with QuantiScan software and the data were normalised to the intensity of the immunostains of the expressed wild-type (WT) α subunits. To verify whether the quantification procedure is correct, oocytes were injected with different amounts of mRNA coding for the ouabain-sensitive rat α2 isoform. The intensity of immunostains of expressed α2 subunits was compared with the number of ^{3}[H]ouabain-binding sites estimated as described in [12]. A linear dependence between the intensities of α2 subunits in Western blots and the number of ouabain-binding sites was observed (data not shown).

3. Results

3.1. Effects of substitutions of Ser-23 on expression and ouabain-specific $^{86}Rb^+$ uptake

To investigate the effects of different substitutions within the α1 polypeptide chain at position 23 on transport function, Ser-23 mutants were expressed in *Xenopus* oocytes and transport activity was assayed as ouabain-sensitive $^{86}Rb^+$ uptake. Fig. 1 shows the expression of the mutants in *Xenopus* oocytes (A) and ouabain-sensitive $^{86}Rb^+$ uptake (B). All Ser-23 mutants were expressed in oocytes, though with somewhat different degree (Fig. 1A). We obtained a stable high expression for the mutant S23E (228 ± 18%, $n = 5$) compared to the WT pump. The expression of mutants S23D and S23A was comparable with those for the WT pump (128 ± 14%, $n = 6$; 80 ± 13%, $n = 4$, respectively). The expression of mutants S23K and S23Q was lower (73 ± 15%, $n = 5$; and 57 ± 10%, $n = 5$, respectively). This was observed for different RNA batches with the same quality of RNA as judged from agarose gels (not shown). Ouabain-specific uptake of $^{86}Rb^+$ normalised to the expression level was considerably lower for the

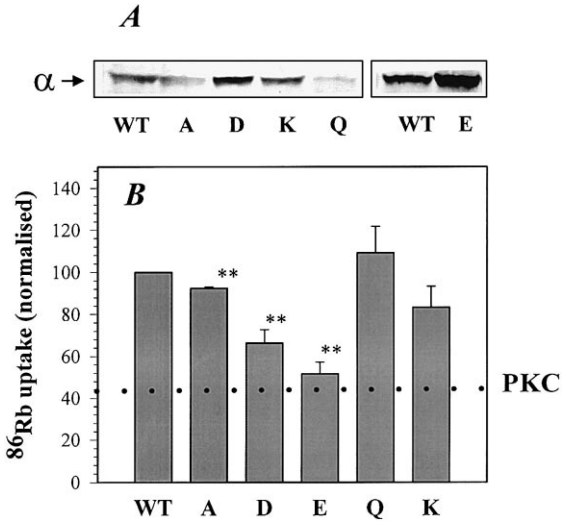

Fig. 1. Effects of replacements of Ser-23 on expression level and normalised ouabain-specific $^{86}Rb^+$ uptake in *Xenopus* oocytes with the expressed Ser-23 mutants. A: Immunostainings of the α1 subunit mutants with anti-α1 antibody. B: Ouabain-specific $^{86}Rb^+$ uptake normalised to the expression degree obtained from averaging the intensities of four–eight immunoblots (five oocytes each) as shown in A. Letters underneath represent corresponding replacements (one-letter nomenclature, WT means wild-type), the dotted line represents the degree of inhibition of $^{86}Rb^+$ uptake in 30 min after injections of the catalytic domain of PKC into oocytes (**$P < 0.01$).

mutants containing the acidic residues Asp or Glu at position 23. In oocytes with the expressed S23E mutant the normalised $^{86}Rb^+$ uptake was reduced nearly to the same degree as in oocytes with expressed WT pumps after injection of the catalytic domain of PKC into the oocytes (see dotted line in Fig. 1B). This result indicates that replacement of Ser-23 by Glu mimics effect of PKC-dependent phosphorylation. If uncharged Gln was introduced at position 23 instead of Glu, $^{86}Rb^+$ uptake was not different from that of the WT pumps. Substitution of Ser-23 by uncharged Ala or positively charged Lys led to only slight reduction of $^{86}Rb^+$ uptake.

3.2. Ouabain sensitivity of the S23E mutant

Since inhibition of transport function was more pronounced for the S23E mutant, this type of pump was investigated in more detail. Fig. 2 shows ouabain dependence of $^{86}Rb^+$ uptake in oocytes injected with RNA for WT and S23E pumps. In both types of oocytes the dependencies of $^{86}Rb^+$ uptake on ouabain concentration exhibit two components. Inhibition at low concentrations with a K_{I1} value of 0.14 μM represents the inhibition of endogenous ouabain-sensitive *Xenopus* pumps. The low affinity component ($K_{I2} = 46.5$ μM) corresponds to the inhibition of ouabain-insensitive α1 rat WT pumps (see also [29]). Substitution of Ser-23 by Glu leads to an extraordinarily ouabain-insensitive pump variant with $K_{I2} = 1.05$ mM.

3.3. Sensitivity to external K^+

Ouabain preferentially interacts with the E2P form of the pump molecule [18] and this is counteracted by external K^+, which drives the pump from the E2P to the E1 form. From the fact that the S23E has a reduced sensitivity to ouabain, one can expect that this may be a result of increased affinity of

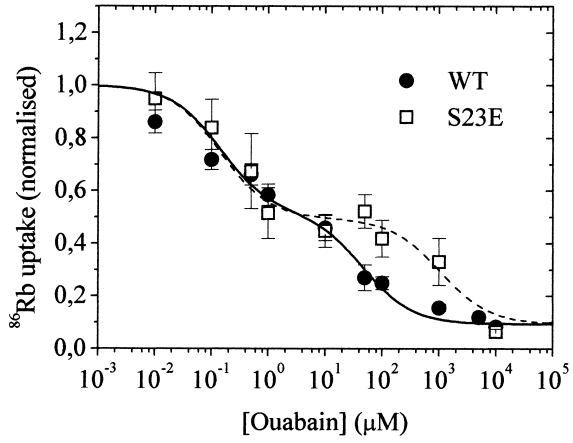

Fig. 2. Dependence of $^{86}Rb^+$ uptake on ouabain in oocytes injected with RNA coding for the wild-type pump and S23E mutant. Closed circles, wild-type; open squares, S23E mutant. For each pump variant $^{86}Rb^+$ uptake was normalised to the maximum uptake in the absence of ouabain. Shown are the averaged data (±S.E.M.) of 10 to 30 measurements. The solid and dotted lines represent fits of:

$$\Phi = \Phi_1 \frac{K_{I1}}{K_{I1} + [\text{ouabain}]} + \Phi_2 \frac{K_{I2}}{K_{I2} + [\text{ouabain}]}$$

to the data. K_{I1} represents the apparent K_I value for inhibition of Rb^+ uptake by the endogenous *Xenopus* pump, K_{I2} the K_I values for inhibition of uptake by the WT or S23E pump, respectively.

the enzyme for external K^+. To investigate whether this might be the case, K_m values for stimulation of pump-generated currents by external K^+ were measured for the WT and the S23E pumps. Since the K_m values are potential-dependent [19], the measurements were performed under voltage clamp. Fig. 3 shows the averaged K_m dependencies for the WT pump and the S23E mutant determined at a holding potential of −30 mV. Similarly as shown for ouabain-specific $^{86}Rb^+$ uptake, normalised maximum pump current generated by the

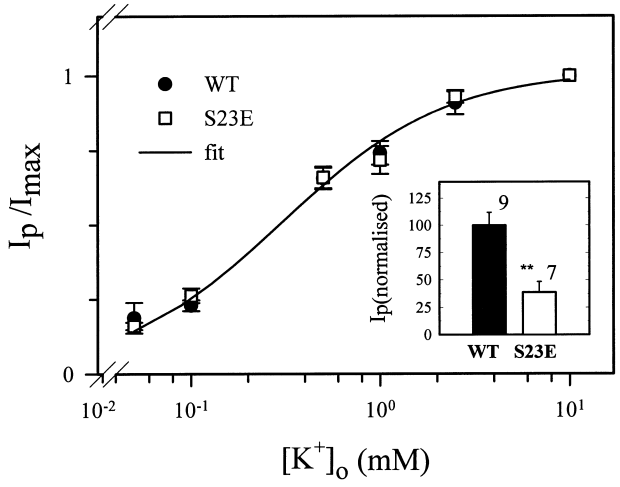

Fig. 3. Dependence of the currents generated by the WT and S23E pumps on external K^+. I_{max}: maximum current generated by each pump variant at 10 mM of $[K^+]_o$. Insert: comparison of I_{max} generated by the WT and S23E (normalised to the expression level and further normalised to I_{max} of the WT). All measurements were done at a holding potential of −30 mV. Shown are averaged data (±S.E.M.) of seven to nine experiments, numbers indicate the number of measurements (**$P < 0.01$). Endogenous pump was blocked with 1 μM ouabain.

S23E mutant is considerably reduced (insert in Fig. 3). However, no difference in K_m values between WT and S23E pumps was observed. Both sets of data can be fitted by the equation $I = I_o*[K^+]_o/(K_m+[K^+]_o)$ with the same $K_m = 0.3$ mM, indicating that the S23E mutant has the same affinity to K_o^+ as the WT pump.

4. Discussion

4.1. Inhibition of transport function

In this work we have demonstrated that substitutions of Ser-23 by acidic residues Asp or Glu reproduce the inhibitory effect of PKC (Fig. 1), which introduces a negatively charged PO_4^{3-} to this position. Replacement of Ser-23 with positively charged and bulky Lys led only to modest inhibition. Gln or Ala did not produce pronounced changes. Hence, the negative charge of the glutamate or aspartate is critical for inhibition of transport function of the Na^+/K^+-ATPase. Inhibition was higher after replacement of Ser-23 with Glu than after substitution with Asp, the acidic residue which is one CH_2 group shorter than Glu, and was not observed after replacement with Gln, which has the same side chain length as Glu. This indicates that the negative charge of Glu at position 23, possibly in combination with a steric hindrance, rather than a simple steric hindrance is responsible for inhibition. Ser-23 is located within the highly charged, hydrophilic and flexible N-terminus. In an earlier work [20] the N-terminus was postulated to function as a cation gate, regulating the access of the cations to their binding sites. Indeed, truncation of 28 residues of the N-terminus including the K-cluster leads to dramatic changes in external cation interactions [11,12]. Further removal of the neighbouring residues up to the T2 site altered the rate of K^+ deocclusion [14], and a tryptic cleavage at the T2 site resulted in a block of the E1→E2 conformational transition [21]. A role of the charged N-terminus as an inactivation gate has been demonstrated directly for the Na^+/K^+-ATPase converted into a channel by palytoxin [22,23]. Jørgensen and Collins suggested that lysines grouped in a cluster within the N-terminus can stabilise transitions between the E1 and E2 conformations of the pump, possibly by formation of salt bridges [21]. Interestingly, the PKC-mediated phosphorylation of Ser-23 shifts the equilibrium between the E1 and the E2 conformation towards the E1 form [7], similar to that observed for the enzyme lacking the N-terminus [21]. Introduction of two negative charges by the phosphoryl moiety in the middle of the K-cluster within the sequence (-*GDKKSKKAKK*-) may, therefore, disrupt the formation of salt bridges formed by the lysines due to electrostatic repulsion with other negatively charged residue(s) within the α subunit protein and lead to a weakening of intramolecular interactions involved in the E1→E2 transition.

Inhibition of function due to mimicry of phosphorylation by negatively charged residues is known for other enzymes [24,25]. Isocitrate dehydrogenase, which is regulated by covalent modification at the active site, could be completely inactivated by the mimicry of phosphoserine-113 by acidic residues [26]. Calculations based on the known structures of the wild-type isocitrate dehydrogenase and the mutants demonstrated that, when a negative charge was introduced either by phosphorylation or site-directed mutagenesis, the change of electrostatic potential was sufficient to inactivate the enzyme. Although a 3-dimensional structure of the Na^+/K^+-

ATPase is not known yet, our data provide useful information for future molecular modelling studies.

The inhibition of transport function of the S23E mutant seems not to be a consequence of reduced sensitivity to extracellular K^+. We could not observe any changes in the apparent K_m for pump stimulation by K_o^+ compared to the wild-type at membrane potential of −30 mV (Fig. 3). This is in contrast to the data reported by Logvinenko et al., who observed a decrease in the apparent activity for K^+ in purified preparations of Na^+/K^+-ATPase phosphorylated by PKC at Ser-23 in vitro [7]. This discrepancy is, most probably, due to differences in the holding potentials used in K_m determination. It is known that the K_m values are potential-dependent, and changes in K_m are less pronounced at negative potentials [16,19]. In the purified preparations of Na^+/K^+-ATPase measurements were done at 0 mV, while in our study at −30 mV, which is close to the physiological resting potential. At this condition alteration in K_m for external K^+ seems to play a minor role.

4.2. Sensitivity to ouabain

The acidic replacement of Ser-23 for Glu within the cytoplasmic N-terminus leads, in addition, to dramatic changes in sensitivity of the receptor site of the external inhibitor ouabain. The changes in K_I are most likely associated with changes in the affinity of the receptor site itself rather than with conformation-dependent changes in apparent ouabain sensitivity due to the moving of the enzyme out of the ouabain-binding E2P conformation by K_o^+. The local perturbations within the N-terminus due to phosphorylation may be most easily protruded up to the first ectodomain and may lead to rearrangements within the receptor site, which is known to be formed by at least two residues in the first transmembrane segment, Cys-104 and Tyr-108 [27,28], and by the residues bordering the first ectodomain Gln-112 and Asn-123 [29]. Affinity to ouabain may be influenced by the cytoplasmic domains of the Na^+/K^+-ATPase located distantly from the receptor site. A drastic effect on sensitivity to ouabain resulting from modification of cytoplasmically orientated C-terminus of the α subunit was recently demonstrated for a chimeric protein consisting of the entire α subunit of the Na^+/K^+-ATPase and the Ca^{2+}/calmodulin-binding domain of Ca^{2+}-ATPase [30].

4.3. Effect of phosphorylation mimicry on the expression of mutants

In addition to the inhibition of transport function, a stable expression exceeding that of the wild-type was observed in oocytes injected with RNA coding for S23D and S23E mutants. Our explanation is that this effect may occur either on the nucleotide or on the protein level. On the nucleotide level substitution of the codons may lead to a stabilisation of RNA or to a stimulation of translation. On the protein level the enhanced synthesis of the α subunit may be interpreted as a result of modification of the polypeptide chain by the acidic replacements or phosphorylation. A stimulated synthesis of proteins phosphorylated by PKC is known for channel proteins. A long-lasting treatment of oocytes with a PKA activator resulted in phosphorylation of the K^+-channel and in an enhanced expression of the K^+-channel protein [31]. The authors explain this phenomenon by PKA-induced stimulation of other cellular proteins, which are responsible for the enhanced synthesis of the channel. The Na^+/K^+-ATPase inhibited by non-toxic doses of ouabain has been demonstrated to initiate a hypertrophic growth of neonatal cardiac myocytes [32]. PKC and Ca^{2+} were involved in the signalling pathways, which led to transcriptional regulation of genes that are markers of cardiac hypertrophy. In our study we did not stimulate PKC in oocytes expressing S23E or S23D, but nevertheless we observed stimulation of the α subunit synthesis. Mimicry of Ser-23 phosphorylation by acidic residues may, therefore, serve itself as a signal for an adapter protein, which may bind to phosphorylated ATPase and may stimulate the downstream protein(s) involved either in stabilisation of mRNA or in the translation machinery leading to an elevated protein synthesis. The enhancement of the α1 subunit protein synthesis after substitutions that mimic the PKC-mediated inhibition of transport function may serve as a concomitant mechanism of adaptation to a long-term stimulation of PKC.

In conclusion, the mimicry of PKC-mediated inhibition of transport function by replacement of PKC phosphorylation site, the Ser-23, with acidic residues demonstrates a crucial role of changes in electrostatic interactions resulting from insertion of a negative charge within the N-terminus of the α subunit by phosphorylation. The S23E and S23D mutants may serve as useful tools for further investigations of the molecular mechanisms of covalent regulation of the Na^+/K^+-ATPase by PKC.

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